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ORIGINAL RESEARCH PAPER

# Biofilm formation by bacterial contaminants of fuel ethanol production

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**Abstract** Commercial fuel ethanol production facilities were previously shown to have characteristic populations of bacterial contaminants which reduce product yield and are difficult to eradicate. Bacterial contaminants were found, for the first time, to form biofilms under laboratory conditions. Fermentor samples from a commercial fuel ethanol production facility were used to inoculate a biofilm reactor and purified bacterial isolates were identified. Biofilms were composed of many of the same species present in production samples, with lactic acid bacteria predominating.

**Keywords** Bacterial contamination · Biofilm · Fuel ethanol · *Lactobacillus* · Wet mill

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## Introduction

Unlike beverage alcohol, fuel ethanol is not produced under pure culture conditions. Chronic infections are expected and tolerated, although they are generally believed to be deleterious to ethanol production (Makanjuola et al. 1992; Narendranath et al. 1997). Contaminants create a constant drain on carbon available for conversion to ethanol and compete for growth factors needed by yeast. They also produce byproducts that are inhibitory to yeast, particularly lactic and acetic acids (Narendranath et al. 2001; Thomas et al. 2002). Acute infections occur unpredictably and can lead to "stuck" fermentations, requiring that facilities be shut down for cleaning, resulting in expensive down times. Penicillin and virginiamycin are commercially sold to treat bacterial infections of fuel ethanol fermentations, and some facilities use these antibiotics prophylactically (Hynes et al. 1997; Bayrock et al. 2003).

It is generally believed that lactic acid bacteria are the primary bacterial contaminants of fuel ethanol fermentations (Chang et al. 1995; Connolly 1999). Production facilities routinely monitor lactic and acetic acid concentrations as a practical means to judge the level of contamination. However, few quantitative identification studies have appeared. In the first ever survey on bacterial contaminants of corn-based fuel ethanol production, we found that individual

facilities tend to exhibit characteristic population profiles over a nine month period (Skinner and Leathers 2004). These results suggested the occurrence of persistent endemic infections typical of biofilms. Consequently, we tested the ability of these contaminants to form biofilms under laboratory conditions.

## Materials and methods

### Treatment of samples

Eight samples (50–100 ml) were obtained from fermentation tanks of a continuous wet mill production facility and immediately stored on wet ice and then at 4°C. Dilutions were made in deMan-Rogosa-Sharpe (Difco MRS, Becton Dickinson, Sparks, MD) broth and plated in duplicate onto MRS plates supplemented with 0.001% cycloheximide (Sigma) to suppress yeast growth. Plates were incubated anaerobically at 37°C using the BBL GasPak Anaerobic System (Becton Dickinson) as previously determined (Skinner and Leathers 2004). At the same time, samples were used to inoculate a biofilm reactor as described below. Random colonies were single-colony isolated three times before identification.

### Bioreactor conditions

A jacketed CDC biofilm reactor (Biosurface Technologies Corp., Bozeman, MT) equipped with stainless steel sample coupons and with a working volume of 350 ml was filled with MRS medium supplemented with 0.05% (v/v) Antifoam A (Sigma) and equilibrated at 37°C with $N_2$ sparging. The reactor was inoculated with 1 ml fresh fermentor sample and incubated for 17 h with stirring at 100 rpm. The reactor was then shifted to continuous flow operation for biofilm growth, with fresh medium pumped through the reactor at 29 ml/min with stirring at 180 rpm.

### Phenotypic identification of bacterial contaminants

Biofilm reactor coupons were sampled as recommended (Heersink 2003), and isolates from the production sample and biofilm reactor were grown on solid MRS medium and identified using both the API 50 CHL test kit (bioMerieux, Montreal, Quebec, Canada) and the Biolog system (Hayward, CA, USA) as previously described (Skinner and Leathers 2004). When identifications based on the two methods did not agree, the identification with the higher confidence value was chosen.

### Sequence identification of bacterial contaminants

Selected bacterial isolates were identified by comparing ribosomal RNA gene sequences to known sequences (GenBank, National Center for Biotechnology Information, Bethesda, MD). A 1.6 kb segment of the 16S rDNA was amplified from genomic DNA (DNEasy kit, Qiagen, Carlsbad, CA) or from cells of a single colony per reaction, using eubacterial primers and PCR conditions described by Whitehead and Cotta (2001). A cycle sequencing kit (Applied Biosystems, Foster City, CA) was used to sequence the amplified product either directly or after cloning (pCR2.1TOPO, Invitrogen Life Technologies Corp, Carlsbad, CA). The nucleotide sequence, which in most cases spanned the entire amplified region, was obtained from both ends of the PCR product. Sequences were analyzed using the BLASTN program (Altschul et al. 1997).

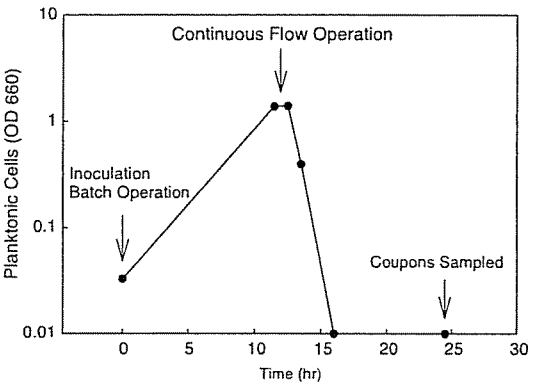


**Fig. 1** Biofilm reactor operations

**Table 1** Species in production and biofilm samples as determined by rapid phenotypic identification[a]

| Species | Number of isolates in production samples | Number of isolates in biofilm samples |
|---|---|---|
| *Actinomyces meyeri* | 0 | 1 |
| *Bacteroides forsythus (Tannerella forsythensis)* | 6 | 3 |
| *Bacteroides fragilis* | 0 | 2 |
| *Bifidobacterium animalis* | 1 | 0 |
| *Bifidobacterium bifidum* | 0 | 3 |
| *Bifidobacterium merycicum* | 1 | 1 |
| *Clostriduim aerotolerans* | 0 | 1 |
| *Clostriduim cellulolyticum* | 1 | 0 |
| *Clostridium clostridiiforme* | 1 | 3 |
| *Clostridium cocleatum* | 0 | 3 |
| *Clostridium tertium* | 1 | 0 |
| *Lactobacillus acidophilus BGB* | 1 | 0 |
| *Lactobacillus amylovorus* | 1 | 7 |
| *Lactobacillus brevis 3* | 2 | 1 |
| *Lactobacillus crispatus* | 1 | 4 |
| *Lactobacillus delbrueckii* subsp. *bulgaricus* | 9 | 0 |
| *Lactobacillus delbrueckii* subsp. *delbrueckii* | 4 | 0 |
| *Lactobacillus delbrueckii* subsp. *lactis* | 1 | 3 |
| *Lactobacillus fermentum* | 2 | 11 |
| *Lactobacillus hamsteri* | 0 | 3 |
| *Lactobacillus hilgardii* | 0 | 1 |
| *Lactobacillus kefiri* | 0 | 1 |
| *Lactobacillus murinus/paracasei* subsp. *tolerans* | 0 | 1 |
| *Lactobacillus oris/parabuchneri* | 1 | 2 |
| *Lactobacillus plantarum 1* | 2 | 5 |
| *Lactobacillus reuteri* | 6 | 2 |
| *Lactobacillus salivarius* subsp. *salicinius* | 1 | 0 |
| *Lactobacillus vaginalis* | 2 | 13 |
| *Lactobacillus* sp. | 2 | 3 |
| *Lactococcus lactis* subsp. *lactis* | 2 | 9 |
| *Lactococcus raffinolactis* | 0 | 1 |
| *Leuconostoc citreum* | 3 | 8 |
| *Leuconostoc lactis* | 1 | 0 |
| *Leuconostoc mesenteroides* subsp. *cremoris* | 16 | 0 |
| *Pediococcus parvulus* | 0 | 3 |
| *Propionibacterium acne* | 4 | 4 |
| *Propionibacterium propionicus* BGA | 1 | 0 |
| *Propionibacterium propionicus* BGB | 7 | 1 |
| *Propionibacterium* sp. | 1 | 0 |
| *Ruminococcus hansenii* | 1 | 0 |
| *Ruminococcus torques* | 0 | 1 |
| No identification | 49 | 35 |
| Total | 116 | 129 |

[a] Isolates identified using the Biolog and API systems

## Results and discussion

### Biofilm formation by contaminants

Following inoculation, biofilm reactors are initially operated in a batch culture phase to promote adhesion of cells to the surfaces of the sample coupons, followed by a continuous flow phase that ensures washout of planktonic cells and permits biofilm growth (Heersink 2003). Figure 1 illustrates the growth and washout of planktonic cells during the biofilm reactor operation as estimated by optical density. In preliminary experiments, 24 h batch cultures produced extravagant biofilms that coated all surfaces and made manipulations difficult. As shown, shorter batch growth phases of about 13 h produced turbid cultures of approx. 1 $OD_{660}$.

Microscopic examination of these cultures showed a mixture of actively budding yeast cells and primarily rod-shaped bacterial cells. A relatively rapid flow rate of approx. 29 ml/min was used in the continuous flow phase to produce a short residence time of just over 13 min, which was empirically determined to be necessary for ensuring washout conditions of rapidly growing bacteria. As shown, the optical density fell to low levels during 3 h of continuous flow operation. Coupons were sampled after an additional 9 h of growth. In the example shown, coupons contained an average cell density of $\log_{10}$ (c.f.u/cm$^2$) of 6.04. Longer incubation periods produced heavier biofilms of between 8.37 and 10.96 $\log_{10}$ (c.f.u/cm$^2$).

## Identification of organisms

Two hundred and forty-five single-colony isolates were purified from production sample inocula and biofilm reactor coupons and preliminarily identified using rapid phenotypic methods as described. These methods identified approx. two-thirds of isolates (Table 1). Consistent with our previous study, the majority of contaminants from the fermentor production samples were species of lactic acid bacteria, with *Lactobacillus* predominating (Skinner and Leathers 2004). Corresponding biofilms were composed of many of the same species (Table 1). Several species, including those tentatively identified as *Lactobacillus amylovorus*, *L. fermentum*, and *L. vaginalis*, were somewhat more abundant in biofilm samples and it is possible that their growth is favored on surfaces. Conversely, a few species were less abundant in biofilms, such as *Lactobacillus delbruekii* and *Leuconostoc mesenteroides*. Overall however, biofilms showed a diversity of species that generally reflected the inocula. It is thus possible that contaminants persist in production facilities as biofilms that are resistant to cleaning and antibiotics. Besides the immediate surfaces of fermentor tanks, there are many other potential sites for biofilm formation, including pipes and heat exchangers.

Several bacterial isolates, including some that could not be identified by phenotypic methods, were identified by sequencing (Table 2). Most of the unknown isolates were identified as *Staphylococcus* and *Clostridium* species, although two were identified as *Lactobacillus panis*. Interestingly, seven biofilm isolates previously identified as various species of lactic acid bacteria were identified as *Lactobacillus amylovorus* by sequencing (Table 2). Phenotypic identifications are determined by descriptive databases that thus far do not include fuel ethanol isolates. Bacteria from fuel ethanol facilities may be phenotypically

**Table 2** Comparison of rapid phenotypic identification and sequence identification of selected isolates

| | Phenotypic identification[a] | Sequence identification[b] |
|---|---|---|
| *Production isolates* | | |
| P2 | *Lactobacillus reuteri* | *Lactobacillus pontis* |
| P22 | *Bacteroides forsythus* | *Propionibacterium acne* |
| P24 | Unidentified | *Staphylococcus* sp. |
| P25 | Unidentified | *Staphylococcus* sp. |
| P29 | Unidentified | *Lactobacillus panis* |
| P32 | Unidentified | *Lactobacillus panis* |
| *Biofilm isolates* | | |
| B3 | *Lactobacillus vaginalis* | *Lactobacillus pontis* |
| B5 | Unidentified | *Clostridium xylanolyticum* |
| B6 | *Lactobacillus crispatus* | *Lactobacillus amylovorus* |
| B11 | *Lactobacillus crispatus* | *Lactobacillus amylovorus* |
| B12 | *Leuconostoc citreum* | *Lactobacillus amylovorus* |
| B13 | *Leuconostoc citreum* | *Lactobacillus amylovorus* |
| B34 | *Leuconostoc citreum* | *Lactobacillus amylovorus* |
| B35 | *Lactobacillus crispatus* | *Lactobacillus amylovorus* |
| B39 | *Lactobacillus crispatus* | *Lactobacillus amylovorus* |
| B42 | *Lactobacillus amylovorus* | *Lactobacillus amylovorus* |
| B49 | Unidentified | *Clostridium xylanolyticum* |
| B50 | Unidentified | *Clostridium xylanolyticum* |

[a] Isolates identified using the Biolog and API systems

[b] Sequence similarities all >99%

atypical, and therefore more difficult to identify by these methods. The abundance of *L. amylovorus* isolates may also suggest that biofilms are less diverse than indicated by phenotypic identifications.

**In summary**, we have established for the first time that bacterial contaminants of commercial fuel ethanol production can form biofilms under laboratory conditions. Results may explain why contamination is persistent and difficult to control in commercial plants. Biofilms are generally regarded as resistant to cleaning and antibiotics (Gilbert and Brown 1995). *Lactobacillus* specifically has been shown to exhibit reduced antimicrobial susceptibility in biofilms (Stewart et al. 2004). New approaches may be needed to control this contamination. Studies are underway to assess the antimicrobial susceptibility of *Lactobacillus* species isolated from commercial ethanol plants.

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